IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Antoniou et al.
Serial No.: 09/247,054 **Group Art Unit: 1632**

Filing Date: February 9, 1999 **Examiner: A. Baker**

For: Self-Replicating Episomal Expression Vectors Conferring Tissue-Specific Gene Expression

DATE OF DEPOSIT:
I HEREBY CERTIFY THAT THIS PAPER IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST CLASS MAIL, POSTAGE PREPAID ON THE DATE INDICATED ABOVE AND IS ADDRESSED TO APPLICATION PROCESSING DIVISION'S CUSTOMER CORRECTION BRANCH, ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, DC 20231.

TYPED NAME: **Doreen Yatko Trujillo**
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Assistant Commissioner for Patents
Washington DC 20231

DECLARATION OF DR. ROBERT CROMBIE

1. I am currently Group Leader of Technology Exploitation for Cobra Therapeutics Limited ("Cobra").
2. I have been with Cobra since 1996.
3. I received my doctorate from the University of Glasgow in molecular oncology in 1993.
4. My curriculum vitae is attached as Exhibit A.
5. I am intimately familiar with the technology involved in the above-identified application, namely locus control region ("LCR") technology. LCR technology is handled by my group.
6. I have read the Final Rejection dated October 4, 2000, and I participated in the interview

conducted on December 12, 2000. I respectfully disagree that the above-identified application is obvious over the references cited by the Examiner.

7. Sadelain et al. report on the use of core sequences of the β-globin LCR in retrovirus mediated gene transfer under conditions in which one copy of the transcription unit per cell is integrated. Sadelain et al. report that when the LCR is so minimized, i.e., reduced to three of the core sequences, position-independent expression became copy number dependent. Sadelain et al. used various sense and antisense combinations of the three core sequences HS2, HS3, and HS4.

8. Contrastingly, Applicants achieved high levels of expression with just these three core sequences, as well as with various combinations of two of these core sequences, as well as with HS2 and HS3 alone. (See page 43 of the application as filed, as well as Figures 4 and 6.)

9. Further, Sadelain et al. observe that "it is important to carefully reevaluate the characteristics of larger LCR-containing sequences in the context of **single**-copy insertions " (page 6732, emphasis supplied). Sadelain et al. then notes importance of obtaining position-independent expression with **single** copy insertions for effective gene therapy.

10. Svennson et al. describe using skeletal muscle as the target for gene therapy. LCRs are not even mentioned and Svennson et al. do **not** describe a self-replicating episomal vector.

11. Thus, the references cited by the Examiner do not show or suggest using LCRs with an episomal vector to achieve tissue specific expression.

12. In that regard, as discussed with the Examiner during the interview conducted December 12, 2000, while Figure 5 of the application as filed may suggest that expression using HS2 alone or HS2, HS3, and HS4 may not be tissue specific, follow-up experiments confirm that they are.

13. As reported in th draft manuscript attached as Exhibit B, and as specifically depicted in Figures 2B and C to the manuscript, attached as Exhibit C, tissue-specific reproducible expression was achieved using HS2 and HS3 of the β-globin LCR alone in a self-replicating episomal vector, as well as when using a combination of HS2. HS3, and HS4. As shown in Figure 2C, expression above background was obtained in the hematopoietic K562 cells using HS2 and HS3 alone, or HS2, HS3, and HS4, but no expression above background was obtained in HeLa cells using these core sequences.

14. The beneficial effects of including LCRs in self-replicating episomal vectors are not limited to the β-globin LCRs. As evidenced by the data presented in Exhibit D attached hereto, Applicants have achieved long term reproducible expression using the CD2 LCR in a self-replicating episomal vector.

15. Specifically, Applicants expressed the gene for enhanced green fluorescent protein (EGFP) in an episomal vector.

16. The p220.EGFP vector was derived from p220.2 (see Figure 1 of the application as filed), an Epstein-Barr virus-based replicating episomal vector containing the EBNA-1 gene and the complete *ori P*, comprising the family of repeats and the dyad symmetry region where replication is initiated. The vector also contains hygromycin and ampicillin resistance genes, a prokaryotic origin of replication and a small multiple cloning site. The expression cassette containing the CMV promoter, gene for EGFP (enhanced green fluorescent protein) and the SV40 polyadenylation signal from pEGFP-N1 (Clontech) was cloned into p220.2 to produce p220.EGFP.

17. The CD2 LCR sequence was taken from vector pVApuro (obtainable from Dr M Antoniou) and cloned into the *Sal* I site of p220.EGFP 5' to the expression cassette and the resultant vector was termed p220.CD2LCR.

18. Human T cell-derived Jurkat cells were electroporated in 2x Optimix buffer (EquiBio). Subsequently the cells were cultured in RPMI with 10% fetal calf serum. After 24 hours

hygromycin B was added at 400 $\mu g\ ml^{-1}$ to select transfectants.

19. Expression of EGFP was analysed by Becton Dickinson FACScan. Cells were given fresh medium on the day preceding analysis. To compare expression levels over a time course, a marker region (M1, see Exhibit D) was set that contained the original EGFP-expressing population of cells measured soon after transfection.

20. The expression of EGFP with and without the presence of an operably linked CD2 LCR was compared (see Exhibit D). Although initial expression levels were similar (see M1 gates for both populations), expression was gradually lost in the absence of the LCR . The Figure shows that long-term expression (here measured at 76 days) was greatly enhanced by the presence of the LCR, despite the maintenance of antibiotic selection and hence the continued retention of the plasmid in both cases. In this case, over 99% of LCR-containing cells maintained long-term high-level of expression, compared with less than 20% of non-LCR-containing cells.

21. Despite the episomal nature of the vectors, the CD2 LCR was capable of exerting a clear effect of promoting sustained, tissue-specific expression of an operably-linked transgene. This is in agreement with the data relating to the β-globin LCR presented in the specification of the above-identified application and in this declaration.

22. I hereby declare that all statements made herein are of my own knowledge true and statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so

made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

4/4/01
Date

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Date of Birth: July 31st 1966 **Citizenship:** Britain

Professional Qualifications:

1988-1993 **Ph.D. Molecular Oncology**
The Beatson Institute for Cancer Research, Faculty of Medicine, University of Glasgow, Scotland

1984-1988 **B.A. Moderatorship (Honours) Genetics**
Department of Genetics, Trinity College Dublin, Ireland

Work Experience:

1996-Present **Group Leader-Technology Exploitation**
Cobra Therapeutics Ltd, Keele, Staffordshire, England
www.cobrat.com
-Manage group of 6 senior research scientists
-Development of Cobra's proprietary gene expression technology (inventor)
-Marketing of technology for out-licensing opportunities
-Partnering of technology
-Investor presentations for round of financing
-Successful applications for substantial industry/academia link awards both MRC-DTI (Cell Engineering LINK Programme) and BBSRC (Gene Technologies Underpinning Health Care)

1993-1996 **Howard Hughes Research Associate**
Baylor College of Medicine, Houston, Texas. USA,
Laboratory of Professor Allan Bradley
-Functional genomics utilising the transgenic "knock-out" approach of gene targeting in mouse embryonic stem cells

1988-1993	**Graduate Student** The Beatson Institute for Cancer Research, Glasgow, Scotland, Laboratory of Professor Allan Balmain Thesis: "The role of Harvey-ras in mouse skin tumorigenesis" - Molecular analysis of the genetic events associated with late stage progression in mouse skin tumours

Scholarships and courses:

	Management Training Courses
Sept. 1997	Fielden House – Management development programme
Dec. 1998	JRK Consultants – Appraisal skills workshop
1987	Awarded the **Irish American Foundation Scholarship** to enable undergraduate research at Section of Genetics and Development, Cornell University, Ithaca, N.Y.

Patent Applications - Presentations - Publications:

R. Crombie and M.Antoniou
International Patent Application, PCT/GB99/02357;WO 00/05393
A polynucleotide comprising a Ubiquitous Chromatin Opening Element (UCOE)

R. Crombie
Oral Presentation at 4th International Gene Delivery and Cellular Protein Expression Conference, Lake Tahoe, California, 21st October 1999

R. Crombie, M. Griffiths, T. Mulcahy, T. Mustoe, S. Williams, E. Yague. and A. Mountain
L. Harland, J. Holdstock and M. Antoniou
CpG islands associated with housekeeping genes prevent chromatin-induced silencing and confer stable transgene expression (Nature Genetics - manuscript submitted)

Y. Mishina, **R. Crombie**, A. Bradley and R. R. Behringer
Multiple Roles for Activin-Like Kinase-2 Signaling during Mouse Embryogenesis
Developmental Biology (1999) 213, 314-326

S. Frame, **R. Crombie** and Allan Balmain
Epithelial carcinogenesis in the mouse: correlating the genetics and the biology. Phil. Trans. R. Soc. London. (1998) 353, 839-845

G.Portella, J.Liddell, **R.Crombie**, S.Haddow, M.Clarke, A.B.Stoler and A.Balmain
Molecular mechanisms of invasion and metastasis during mouse skin tumour progression. Invasion and Metastasis (1994-95) 14:7-16